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13. ABSTRACT *(Maximum 200 Words)*

EGFRvIII is a ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has detected in many human cancers, including prostate cancer, but has never been detected in normal tissue. In this report, we demonstrated that 35% (74/208) prostate cancer patient specimens detected EGFRvIII-co-expression with ErbB-2. To delineate the biological significance of EGFRvIII in human prostate cancer, we expressed EGFRvIII in Tsu and Du145 prostate cancer cells. Expression of EGFRvIII in Tsu and DU145 cells revealed enhancement of proliferation *in vitro* and increased tumorigenicity in nude mouse. We also designed and generated a tumor specific ribozyme targeted at the fusion junction of EGFRvIII. This specific EGFRvIII ribozyme is able to effectively cleave EGFRvIII mRNA under physiological conditions in a cell-free system. While expressing this EGFRvIII-ribozyme in 32D/EGFRvIII cell, EGFRvIII-ribozyme is capable of down-regulating EGFRvIII expression. However, this ribozyme has not effect on wild-type EGFRmRNA and protein levels. These results suggested that we have generated a tumor-specific biologically functional ribozyme. These results provide the first evidence that EGFRvIII plays a role in human prostate cancer tumorigenesis.

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INTRODUCTION

Clinical significance of EGFRvIII in human cancer

Prostate cancer is one of the most commonly diagnosed invasive malignancies and is the second leading cause of death in American men (1-3). The epidermal growth factor receptor (EGFR/ErbB) family is a group of tyrosine kinases, frequently overexpressed in a variety of carcinomas (4-6). This class I subfamily is comprised of four members: EGFR (4), *HER2*/p185^{erbB-2}/neu (5), *HER3*/p160$^{erbB\ 3}$(6) and *HER4*/p180 $^{erbB-4}$ (19). Autocrine production of transforming growth factor alpha (TGFα) and overexpression of epidermal growth factor receptor (EGFR) may contribute to androgen-independent prostate cancer growth at both primary and metastatic sites (4). The epidermal growth factor receptor (EGFR) is a promising target for various anti-neoplastic agents. However, normal EGFR may be targeted by these anti-EGFR agents, a more appropriate strategy might be to target an alteration within the receptor. Several reports have documented spontaneous rearrangements within the EGF receptor gene in primary human glioblastoma tumor (7-10). Three different types of mutants result from these rearrangements (8). The most common of these rearrangements is the Type III EGF deletion-mutant receptor (EGFRvIII), which involves a deletion of exons 2-7, (cDNA nucleotide 275-1075), presumably through alternative splicing or rearrangements (11, 12). Overexpression of this mutant EGF receptor in NIH3T3 cells results in transformed morphology, enhanced growth, and tumorigenicity in athymic mice (13). Crosslinking experiments with EDAC demonstrate that the mutant EGF receptor is dimerized in the absence of ligand. EGFRvIII has not been detected in normal adult tissues. Recent reports demonstrated that the EGFRvIII is also frequently detected in human prostate cancer (12, 14). Our preliminary results detected 44% of primary prostate cancer specimens express EGFRvIII. All of these EGFRvIII positive tumors appear to be poorly differentiated with high Gleason grade prostatic adenocarcinomas. This phenomenon is

the first evidence that EGFRvIII expression is detected in high grade human prostate cancer. Furthermore, overexpressing EGFRvIII in a murine hematopoietic IL3-dependent cell line (32D cells) caused acquisition of a ligand-independent and IL-3-independent phenotype and formed large tumors in nude mice (21). However, the tumorigenicity potential of EGFRvIII in prostatic cancer cells has not yet been explored. The understanding of the function and biology of EGFRvIII will have important implications in the prognosis and treatment of prostate cancer.

Ribozymes:

Specific gene modulation, using oligonucleotides, including triplex DNA, antisense DNA/RNA and ribozymes, have been used as strategies for suppressing activated oncogenes (17-20). Ribozymes are catalytic RNAs that are capable of specific strand scission (15, 16). The targeting of specific gene transcripts with ribozyme constructs can successfully inactivate genes produced in cells and can generate dominant negative mutant (15). Hammerhead ribozymes are self-cleaving RNAs whose catalytic activity has been mapped to a small core of less than 40 nucleotides, arranged in a typical three-stem structure (15, 16). The targeted cleavage site is GUX (X is either A, U, or C) (15, 16). Targeted suppression of specific gene expression by ribozymes has been reported (17), including activated ras and truncated bcr-abl gene (19, 20). In our previous studies, we generated a specific ribozyme targeting ErbB-4. We demonstrated that ribozyme-mediated down-regulation of ErbB-4 resulted in reduction of proliferation *in vitro* and inhibition of tumor growth *in vivo* in breast cancer cell lines (18). Ribozymes, therefore, provide an alternative way for modulation of specific gene expression. High frequency of EGFRvIII expression is detected in a wide variety of human tumors, but it is not detectable in normal tissues, therefore, it might be an excellent target for biologically based therapies. Our AIM 3 will use ribozyme strategy targeting the novel fusion junction of EGFRvIII in order to block

EGFRvIII expression in prostate cancer cells so that we may study the effect on tumor progression. In theory, the EGFRvIII ribozyme should be tumor specific and the ultimate goal of this study is to provide proof of principle for a potential translational gene therapy.

In this final report, I will summarize all our findings that we have accomplished during the last three year funding period. We have achieved the goals that were proposed in the proposal.

BODY

Specific Aim 1. To determine correlation of EGFRvIII expression with clinipathological prognostic factors in human prostatectomy specimens:

Aim 1 – Synopsis: We have determined the correlation of EGFRvIII expression with ErbB-2 expression in human prostate cancer and have analyzed its implication for clinical outcome.

Rationale: In our preliminary studies, we demonstrated that EGFRvIII is expressed in 44% of human prostate cancer. Within a given tissue, EGF-family receptors are rarely, if ever, expressed alone, but are found in various combinations. For example, ErbB-2 is a co-receptor and co-expression of ErbB-2 would diversify its signaling. Compelling clinical has data shown that overexpression of EGFR correlates with poor prognosis (5). Therefore, we determined the correlation of EGFRvIII expression with ErbB-2 expression in human prostate cancer.

Results:

Previously, we demonstrated that 44% of prostate cancers express EGFRvIII (Figure 1). To investigate whether any correlation might exist between the ErbB-2 expression and EGRFvIII expression in prostate cancer, we performed immunohistochemical analysis with a specific ErbB-2 antibody on the same set of prostate cancer tissue microarrays. We detected that about 35% (74/208) of prostate cancer specimens express both EGFRvIII and ErbB-2. Within this set of 74 prostate cancer samples, about 28% (20/74) of these cases exhibit high expression levels of both EGFRvIII and ErbB-2 receptors. There are 31% (65/208) of these prostate cancer specimens that were negative for both receptors. Other tumors express either EGFRvIII or ErbB-2 alone. Table I summarizes the percentage of co-expression of EGFRvIII and ErbB-2 in prostate cancer patient specimens.

Table 1. Co-expression of EGFRvIII with ErbB-2 in primary human prostate cancer

Co-Expression status	# of cases	% (N/208)
EGFRvIII- /ErbB-2-	65	31.6
EGFRvIII+ /ErbB-2+	54	26.2
EGFRvIII ++ /ErbB-2++	20	9.7
EGFRvIII++ /ErbB-2-	21	10.2
EGFRvIII+ /ErbB-2-	22	10.7
EGFRvIII- /ErbB-2+	24	11.7

Note: - (negative); + (weak positive); ++ (strong positive)

Figure 1. Illustration of the EGFRvIII expression in prostate cancer specimens.

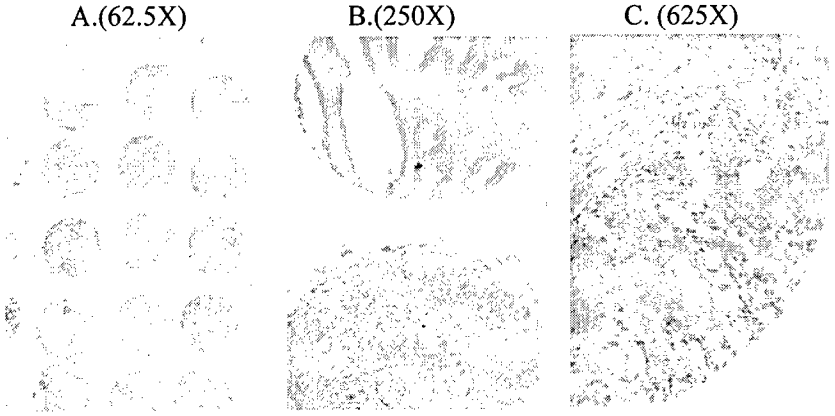
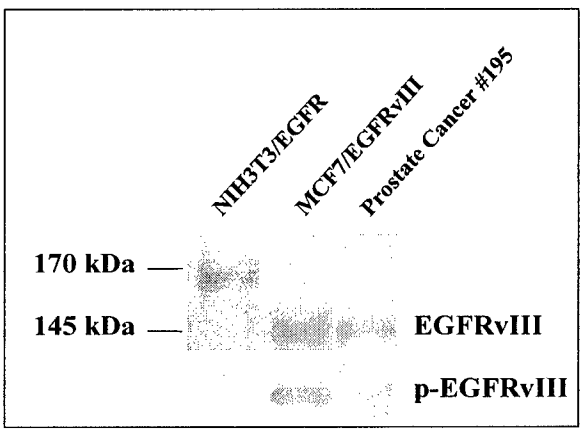


Figure 1: Immunohistochemical analysis of a prostate tissue microarray with EGFRvIII-specific antibody (Ab-5H) counterstained with hematoxylin for viewing negative stained cells (Blue). EGFRvIII positive stained cells appear as brown. A, B and C illustrates the tissue microarray under different magnification.

To further determine whether or not EGFRvIII is phosphorylated in human prostate tumor specimens, we analyzed its autophosphorylation in a set of frozen prostate cancer tissue specimens. As shown in Figure 2, the primary invasive prostate tumor tissues obtain the phosphorylated form of EGFRvIII. This result clearly demonstrated that EGFRvIII is a constitutively activated receptor in human prostate cancer.

Figure 2: Constitutively activated EGFRvIII in human prostate cancer
Lysates from frozen section of prostate tumor specimens were immunoprecipitated with anti-EGFRvIII (4-5H) antibody or with anti-EGFR (528) for NIH/EGFR cells and subsequently western blotting with an anti-phosphotyrosine antibody or anti-EGFR antibody used as a loading control. Bands were visualized using chemiluminescence's detection system.
Lane 1: EGFR transfected NIH 3T3 cells.
Lane 2: EGFRvIII transfected MCF-7 cells.
Lane 3: Prostate cancer specimens #195.



Conclusion: EGFRvIII is constitutively activated in human prostate cancer. Co-expression of ErbB-2 with EGFRvIII may play a role in prostate cancer progression.

Methods:

1) Immunohistochemistry: Immunohistochemistry was carried out on primary prostate tumors tissue microarray with the specific ErbB-2 monoclonal antibody (Ab-3, Oncogene Research Products, MA). Immunoreactivity was scored with both the percentage of cells positive and the overall intensity of the staining was taken into account.

2) Immunoprecipitation and Western blotting analysis: Prior to immunoprecipitation, protein concentration was determined by BCA Protein Assay (Pierce, IL, USA). Tissue extracts were clarified by centrifugation at 12,000rpm, and the supernatants were subjected to immunoprecipitation with anti-EGFR or anti-EGFRvIII antibodies. After overnight precipitation at 4°C, protein A-agarose beads were added and left for a 2h incubation at 4 °C. The immunocomplexes were then separated by SDS-PAGE and transferred to nitrocellulose (Amersham Bioscience, NJ, USA) for western blot analysis. The membrane was blocked with TBST containing 5% nonfat dry milk for 1h at room temperature with constant agitation. Membranes were probed with appropriate primary antibody overnight at 4 °C, washed three times with TBST, and then incubated with secondary antibody for 1 h at room temperature and additionally washed five times. Immunoreactive bands were visualized by an enhanced chemiluminescence reagent (ECL; Amersham Bioscience, NJ, USA), and exposed to film (Amersham Bioscience, NJ, USA).

3) Statistical analysis: Fisher exact test and Pearson's regression-coefficient test were used to evaluate the statistical significance of the results. We consulted with biostatistician Rebecca Slack in Biostatistics Core-facility of the Lombardi Cancer Center.

Specific Aim 2: To test the hypothesis that over-expression of deletion-mutant EGFRvIII is capable of increasing the malignancy of tumor cells.

Aim 2-Synopsis: We transfected the EGFRvIII into prostate cancer cell lines and determined the biological and biochemical effects mediated by EGFRvIII in these transfectants *in vitro and in vivo*.

Rationale: EGFRvIII is a constitutively activated receptor and is expressed in 44% of prostatic adenocarcinomas. Normal tissue and benign prostate tumors do not express EGFRvIII.

This suggests that EGFRvIII expression is highly correlated with prostate cancer progression. Moreover, transfection studies on rodent fibroblasts have shown that over-expression of the EGFRvIII can result in the acquisition of the transformed phenotype (13). In addition, our early studies demonstrated that expressing EGFRvIII in 32D cells abrogated IL-3 dependency of these cells *in vitro* and induced tumor formation *in vivo* (21). However, the tumorigenicity of EGFRvIII in prostate cancer cells has not yet been explored. To accomplish this, we transfected EGFRvIII cDNA into Tsu, DU145 and LNCap prostate cancer cell lines, we then determined the biological consequences of EGFRvIII *in vitro* and *in vivo*. These results will enable us to determine whether EGFRvIII expression involves prostate cancer tumorigenesis.

Results:

Established stable transfected TSU/EGFRvIII and DU/EGFRvIII prostate cell lines

To investigate the biological role of EGFRvIII in prostate cancer cells, we transfected EGFRvIII cDNA in Tsu, DU145 and LNCaP prostate cancer cell lines, stable clones were selected. FACS analysis was performed to identify and quantify the EGFRvIII expression in these transfectants. Figure 3 illustrates the EGFRvIII expression levels in two of the DU-145 transfectants (#12 and #69). FACS analysis revealed that DU-145/EGFRvIII12 (green color curve) expresses relatively lower levels of EGFRvIII, whereas DU145/EGFRvIII69 (Blue color curve) expresses relatively higher levels of EGFRvIII.

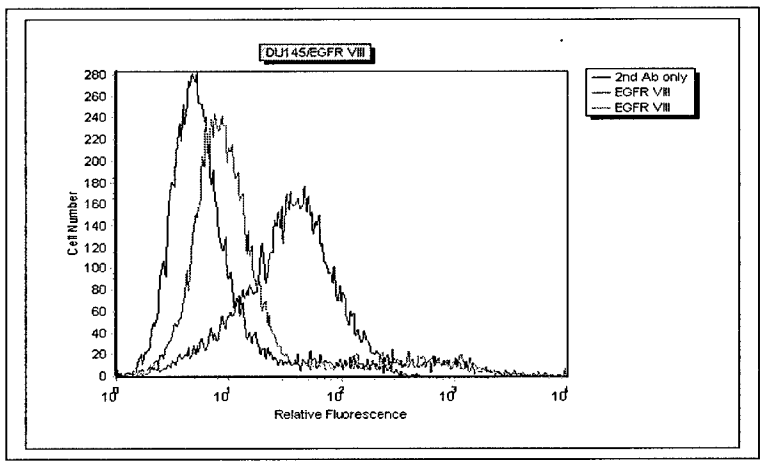


Figure 3. Expression levels of EGFRvIII in DU-145/EGFRvIII transfectants. 1x 10^6 cells were used for FACS analysis. The black (most left) curve represents nonspecific staining (primary antibody omitted). The other line curves (green and blue) represent the expression levels of EGFRvIII in two of the DU145 transfectants #12 and #69.

Furthermore, western blotting with an EGFRvIII antibody (4-5H) was performed to confirm the EGFRvIII expression in these EGFRvIII transfectants. Figure 4 illustrates the EGFRvIII protein levels in Tsu/EGFRvIII cells.

Figure 4 Expression levels of EGFR vIII in TSU transfectants

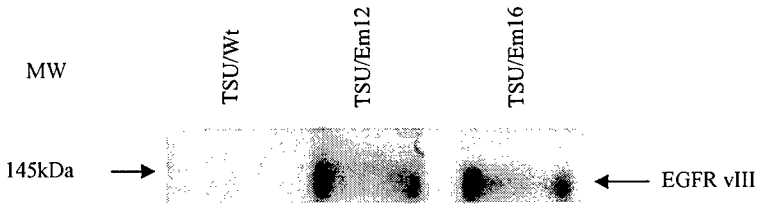


Figure 4. Total cell lysates (30ug) from TSU and TSU/EGFRvIII transfectants were separated by electrophoresis using SDS-PAGE and transferred onto nitrocellulose membranes. Bands were visualized using monoclonal EGFRvIII antibody (4-5H) and a chemiluminescence's detection system.

Constitutively activated EGFRvIII in TSU transfectants

In an effort to better understand the biological role of EGFRvIII in prostate cancer, we further evaluated the autophosphorylation of EGFRvIII by immunoprecipitation with anti-EGFRvIII (4-5H) antibody, subsequently western blotting with a specific phosphotyrosine antibody. As shown in Figure 5, EGFRvIII are constitutively activated in Tsu/EGFRvIII transfectants. Moreover, comparing with the parental cells, expressing EGFRvIII in Tsu cells induces wild-type EGFR phosphorylation. These results suggest that EGFRvIII could activate wild-type EGFR signaling pathways, further indicating that EGFRvIII may heterodimerize with wild-type EGFR and diversify its signaling pathways.

Figure 5 Constitutively activated EGFR vIII in EGFR vIII Transfected TSU Cells

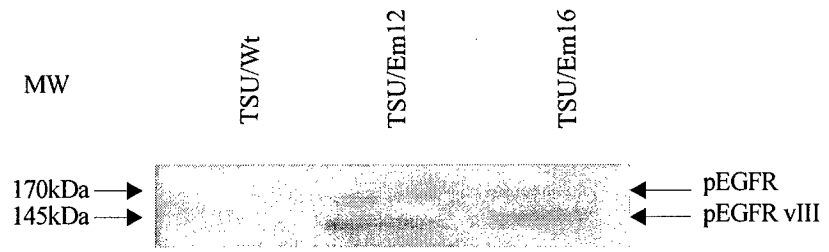


Figure 5. One mg of lysates from various EGFRvIII transfected TSU cells (clone #12 and #16) and the parental TSU cells were immunoprecipitated with a specific anti-EGFR antibody, which recognizes both the wild-type EGFR and the mutant form (EGFRvIII) of EGFR. These precipitated proteins were subsequently subjected to western blotting with an anti-phosphotyrosine antibody. Bands were visualized using a chemiluminescence detection system.

To further confirm these findings, we also assessed the autophosphorylation status of EGFRvIII transfected DU-145 cells. Similar observations were made in DU-145/EGFRvIII transfectants, as illustrated in Figure 6, EGFRvIII is constitutively activated in DU-145/EGFRvIII cells as well.

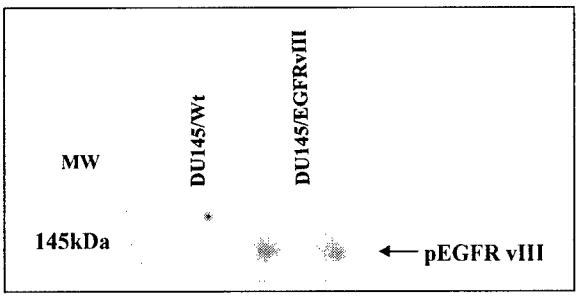


Figure 6. Constitutively activated EGFRvIII in DU-145/EGFRvIII cells. 1mg of lysates from the parental DU145 and EGFRvIII transfected DU145 cells were immunoprecipitated with anti-EGFRvIII (4-5H), in combination with protein A Sepharose CL-4B overnight at 4^0C with gentle agitation. Immunoprecipitates were then separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody, followed by blots with secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (ECL; Amersham Corp.).

These results demonstrate that EGFRvIII is constitutively activated in a ligand-independent manner in prostate transfectants. In addition, expressing EGFRvIII enhances wild-type EGFR phosphorylation, which implies that EGFRvIII could activate wild-type EGFR signaling pathways.

EGFRvIII Enhances Prostate Cancer Cell Proliferation.

To evaluate whether overexpression of EGFRvIII will have any effects on prostate cancer cell proliferation, anchorage-dependent growth assays were conducted to assess and correlate the EGFRvIII expression levels with the rate of proliferation. Three independent experiments were performed in triplicate determining cell numbers on day 2, 4, and 7. Values were reported as the mean of triplicate determinations ± SD. Figure 7 demonstrated that expressing EGFRvIII in TSU prostate cancer cell induces proliferation. Similar observations were also seen in EGFRvIII

transfected DU145 cells as illustrated in Figure 8. These results indicated that EGFRvIII play a role in prostate cancer cell proliferation.

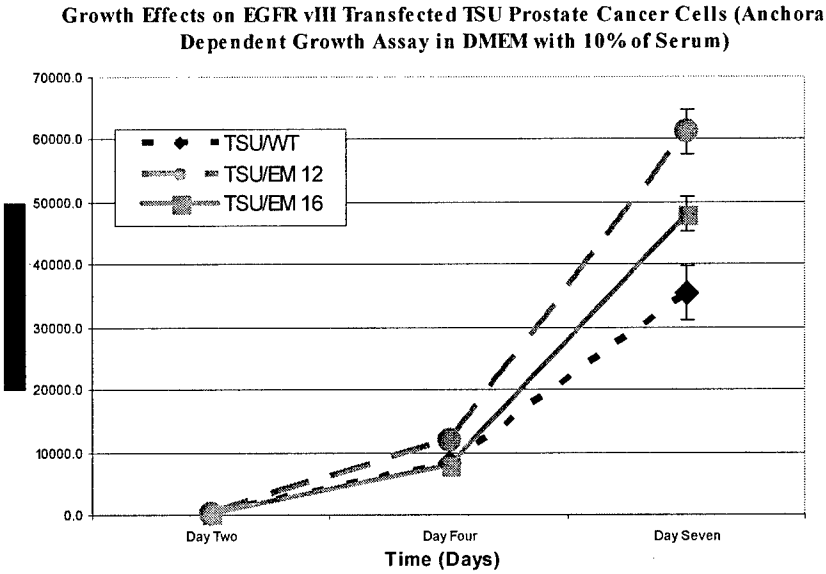


Figure 7. EGFRvIII enhances proliferation in Tsu transfectants. Growth assays were performed on TSU parental cell and EGFRvIII transfected TSU cells. Approximately 1500 cells were seeded in 24-well plates and propagated in 10% FBS. Cells were counted on day 2, 4 and 7. Values were reported as the mean of triplicate determinations ± SD.

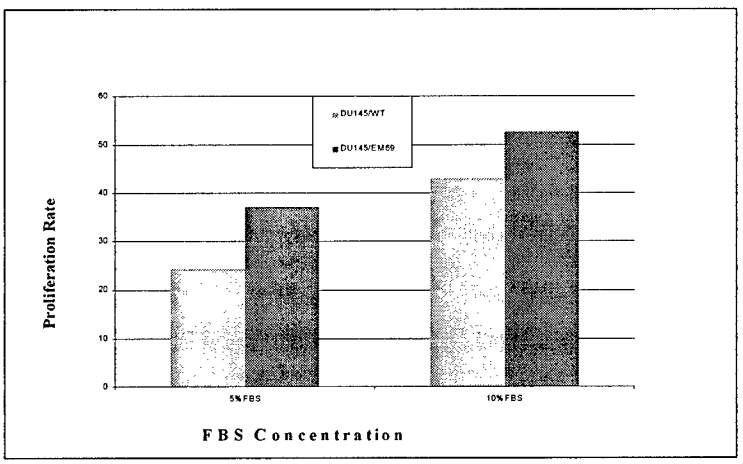


Figure 8. EGFRvIII-mediated proliferation in DU-145/EGFRvIII transfectants. Growth assays were performed on DU145 parental cell and EGFRvIII transfected DU145 cells. Approximately 1500 cells were seeded in 24-well plates and propagated in 5% or 10% FBS. Cells were counted on day 2, 4 and 7. Values were reported as the mean of triplicate determinations ± SD.

Expressing EGFRvIII Enhances Tumorigenicity in vivo.

Because expressing EGFRvIII enhances proliferation of prostate cancer cells *in vitro*, we wondered whether expressing EGFRvIII in prostate cancer cells would be able to increase the malignancy *in vivo*. We explored the potential tumorigenicity of EGFRvIII *in vivo* and as we expected, expressing EGFRvIII in prostate cancer cells enhance tumorigenicity in nude mouse as demonstrated in Figure 9.

Figure 9. Xenografts of Tsu/EGFRvIII transfectants

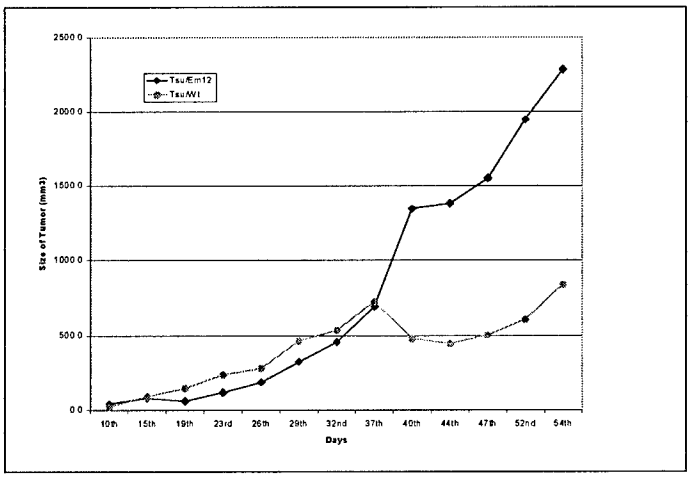


Figure 9. Five x 10^6 EGFRvIII transfected cells and parental cells, as well as vector transfected cells, were injected intraperitoneal in athymic nude mice. TSU/EGFRvIII cells grew large tumors with a mean tumor size of 2300 mm^3, whereas tumors that grew in the parental cells were smaller (800 mm^3).

Conclusion: These results clearly demonstrate that EGFRvIII plays a role in prostate cancer progression.

Methods:

1) Establishing EGFRvIII stable transfected prostate cancer cells: We have obtained the full-length cDNA for EGFRvIII. 15-20μg of plasmid DNA of pcDNA3-EGFRvIII was used for transfection using the calcium phosphate precipitation method into Tsu, DU145 and LNCap prostate cancer cell lines. The individual clones that are resistant to G418 will be selected and expanded for further characterization.

2) Identification and quantization of EGFRvIII expression in Tsu, DU145 and LNCaP transfectants by FACS analysis: Cells (1 x 10^6) were harvested and then stained for 1 hr with anti-EGFRvIII monoclonal antibody (4-5H) at 4 °C. Stained cells were then washed with cold PBS. A secondary FITC-anti-mouse antibody (CALTAG, CA, USA) was used, and the expression levels of EGFRvIII were quantitatively measured by flow cytometry.

3) Evaluation of EGFRvIII autophosphorylation: Cells were lysed in HEPES lysis buffer (50 nM HEPES, 150mM NaCl, 10% glycerol, 1% Triton X 100, 1.5 mM $MgCl_2$, and 1mM EGTA), and the cell debris was pelleted by centrifugation. The lysates were then subjected to immunoprecipitation with anti-EGFRvIII (4-5H), in combination with protein A Sepharose CL-4B (Amersham Pharmacia, Sweden) overnight at 4^0C with gentle agitation. Immunoprecipitates were then separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody (Upstate, Lake Placid, NY), followed by blots with 0.5 ug/ml of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (ECL; Amersham Corp.).

4) Anchorage-dependent growth assays: Cells was harvested after trypsin treatment and 5,000 cells/well was plated in 24-well plates (Costar). All samples were prepared in triplicate. Three independent assays were performed. Cells were counted in a Coulter Counter (Coulter Electronics LTD, Hialeah, FL) on day 2 (the following day), day 4 and day 7. Cell counts will be reported as the means of triplicate determinations ± SD.

5) *In vivo study:* We injected intraperitoneal (5 x 10^6 cells) Tsu/wt, Tsu/EGFRvIII into 4-6 week old male athymic nude mice, using five mice per group. Tumor growth was monitored and the tumor size was measured twice weekly from which we were able to calculate the tumor volume (length x width x thickness).

Specific Aim 3: Generation of tumor-specific ribozyme targeted at the novel fusion junction of EGFRvIII.

Rationale: EGFRvIII is a tumor specific cell surface molecule. A high frequency of EGFRvIII receptor is found in prostate cancers, but is not found in normal prostate tissue (12, 14). These unique features make EGFRvIII an excellent target for biologically based therapies. To investigate whether or not down-regulation of EGFRvIII expression reduces the tumorigenicity and metastasis in human prostate cancer cells, we used molecular targeting of the EGFRvIII mRNA by ribozymes. We generated a tumor-specific ribozyme, targeting the novel junction sequence of EGFRvIII mRNA 5'-AAGAAAG**GUA**AUUAUGU-3', where the bolded, underlined nucleotides comprise the novel cleavage site for this ribozyme. This cleavage sequence created a novel junction with a glycine residue by the joining of EGFR exon I to exon VII. We stably expressed this tumor-specific EGFRvIII ribozyme in human prostate cancer cells to examine the

effect of the abrogation of EGFRvIII expression in prostate cancer cells. The novelty of the EGFRvIII-ribozyme is that it specifically targets the tumor cells, as normal cells do not express this mutant EGFR. In addition, this EGFRvIII ribozyme only targets the EGFRvIII mRNA, not the wild-type EGFRmRNA.

Results:

Generation of EGFRvIII ribozyme targeting the fusion junction of EGFRvIII

In an attempt to elucidate whether down-regulation of EGFRvIII in prostate cancer cells would reduce the tumorigenicity. We generated a specific ribozyme targeting the novel junction sequence of EGFRvIII mRNA 5'-AAGAAAG**GUA**AUUAUGU-3', where the bolded with underlined nucleotides comprise the novel cleavage site for this ribozyme. Thus, this **ribozyme cleaved at the proposed fusion junction of the EGFRvIIImRNA, whereas the wild-type EGFRmRNA which does not contain this 5'-AAGGUAAUU-3' junction sequence was not cleaved by this ribozyme**. The schematic representation of the design of the EGFRvIII ribozyme is shown in Figure 10.

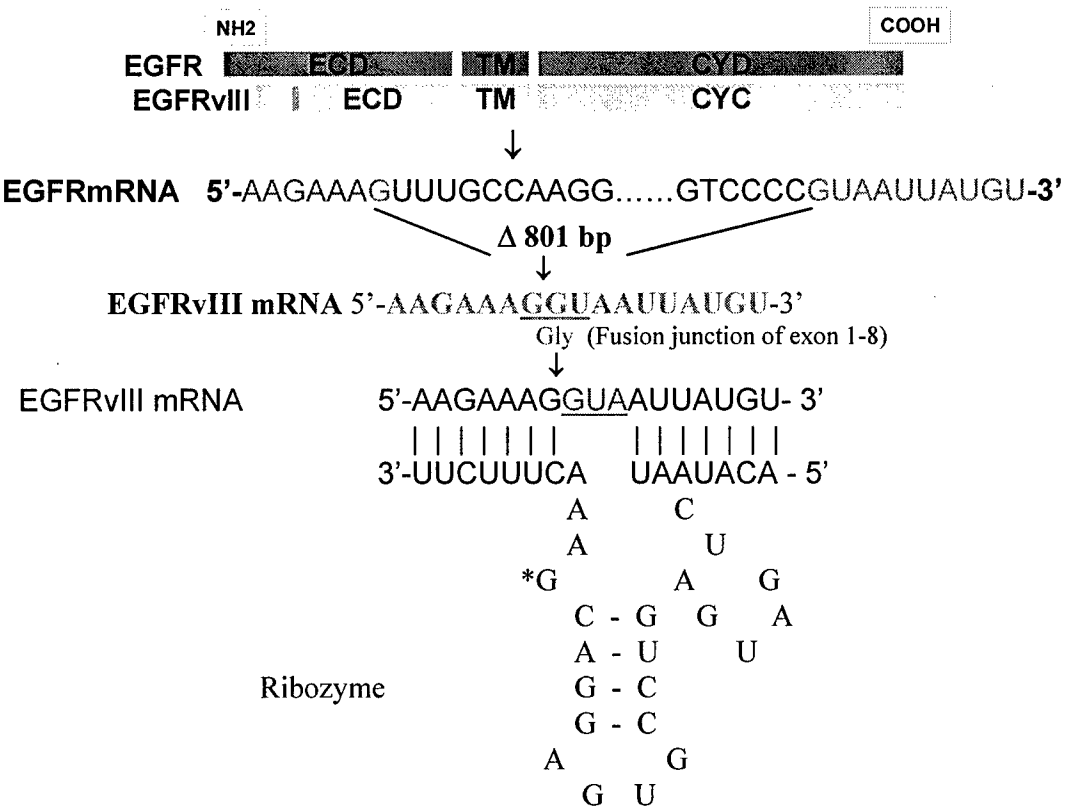


Figure 10. Schematic representation of a specific hammer-head ribozyme targeting the novel junction of EGFRvIII mRNA.

EGFRvIII ribozyme-mediated mRNA cleavage in vitro.

To evaluate the catalytic activity of this ribozyme, we first conducted an *in vitro* run-off transcript to assess the ribozyme-mediated cleavage of EGFRvIII mRNA. As shown in Figure 11, this ribozyme precisely and efficiently cleaves EGFRvIII mRNA.

Figure 11.

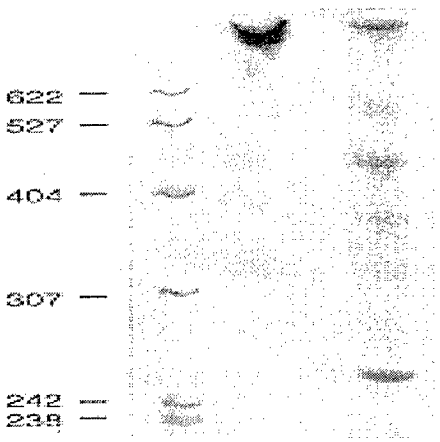


Figure 11: Catalytic activity of EGFRvIII ribozyme in a cell free system. Lane 1 represents molecular weight marker; Lane 2 represents the ^{32}P-labled EGFRvIII transcripts with expected size of 715 nucleotides. Lane 3 represents the predicted cleaved products (455 and 260 nucleotides) by EGFRvIII ribozyme.

Evaluation of the specificity and efficacy of EGFRvIII ribozyme in an intracellular model system (32D cells):

Although ribozyme sensitivity in an extracellular system can be correlated with the predicted secondary structure of the target RNA, the intracellular susceptibility of the target RNAs to ribozymes does not necessarily correlate with their predicted secondary structure. We evaluated the intracellular EGFRvIII ribozyme efficacy and specificity by utilizing the 32D cell system.

The 32D cell is an IL-3 dependent mouse hematopoetic cell line. We observed that overexpression of EGFRvIII in these cells capable of proliferating in the absence of IL-3 (Figure 12) and addition of exogenous EGF werefurther enhanced their growth (Figure 12). In contrast, the parental 32D cells absolutely required IL-3 for growth and were unresponsive to EGF treatment. The wild-type 32D/EGFR cells required either IL-3 or cognate ligands for EGFR for growth. In the absence of IL-3 or lacking of cognate ligands, 32D/EGFR cells did not survive. However, high levels of EGFRvIII were able to abrogate these IL-3-dependent pathways in the absence of IL-3 and absence of ligands. As demonstrated in Figure 12, 32D cells overexpressing EGFRvIII (32D/EGFRvIII P5) were able to survive and proliferate in the absence of IL-3 and in

the absence of ligands (21). Thus, this system provided a simple bio-assay though which ribozyme activity can easily be detected.

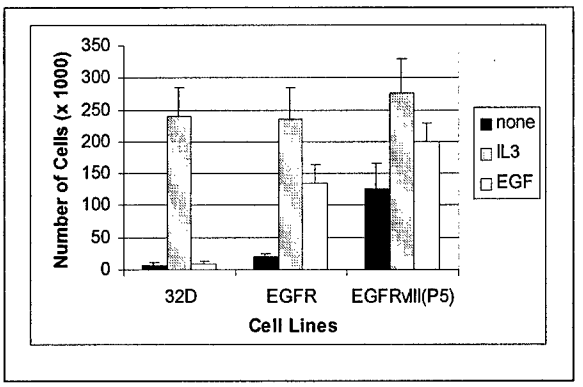


Figure 12. EGFRvIII-mediated IL-3-independent phenotype in 32D/EGFRvIII P5 cells. 32D, 32D/EGFR and 32DEGFRvIII P5 cells were plated at a density of 5 x10^4 cells/ml in IL-3 free medium (none, solid bar), medium supplemented with IL-3 (strike bar), or medium lacking IL-3 but supplemented with 100 ng/ml of human recombinant EGF (open bar). Viable cells were counted on day 3 after seeding. All samples were prepared in triplicate. This assay was repeated more than three times.

EGFRvIII-ribozyme mediated downregulation of EGFRvIII in an intracellular model system:

We constructed EGFRvIII-ribzoyme into *pcDNA3.1/zeo* vector and transfected it into 32D/EGFRvIII cells. The individual clones that are resistant to *zeocin* were selected and characterized. Northern blotting was performed to determine the EGFRvIII mRNA expression in ribozyme transfected 32D/EGFRvIII cells.

Immunoblotting was performed to confirm the ribozyme-mediated down-regulation of EGFRvIII in the transfected cells, as shown in Figure 13. The ribozyme was able to efficiently reduce the EGFRvIII expression levels in 32D/EGFRvIII, which suggests that we have generated a biologically activate EGFRvIII ribozyme.

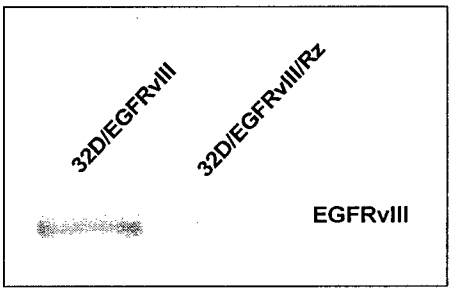


Figure 13. EGFRvIII ribozyme is capable of down-regulating EGFRvIII expression in 32D/EGFRvIII cells. 40 ug of cell lysates from 32D/EGFRvIII and ribozyme transfected 32D EGFRvIII (32D/EGFRvIII/Rz) cells were separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with specific EGFRvIII antibody (4-5H). Immunoreactive bands were detected with an enhanced chemiluminescence's reagent.

The specificity of this ribozyme was determined by transfecting the EGFRvIII-ribozyme into cells expressing wild-type EGFR. The ribozyme effects on wild-type EGFRmRNA and protein levels were then determined by Northern blot and FACS analysis. Figures 14A and 14B demonstrate that specific EGFRvIII ribozyme does not have any effect on wild-type EGFRmRNA and protein levels.

A.

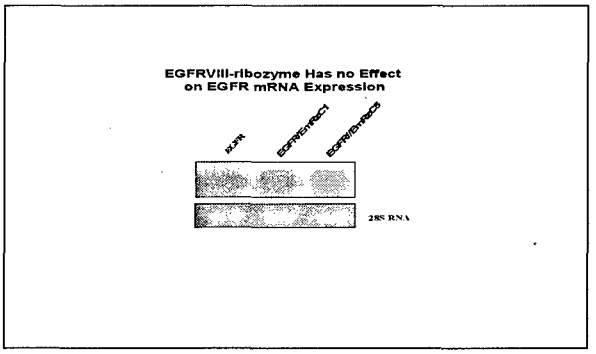


Figure 14A. EGFRvIII ribozyme has no effect on wild-type EGFRmRNA. RNA extracted from EGFR cells and ribozyme transfected cells were subjected to northern blotting with an EGFR probe. Lane 1 is EGFR transfected parental cells. Lane 2 and lane 3 are two ribozyme transfected EGFR expressing clones.

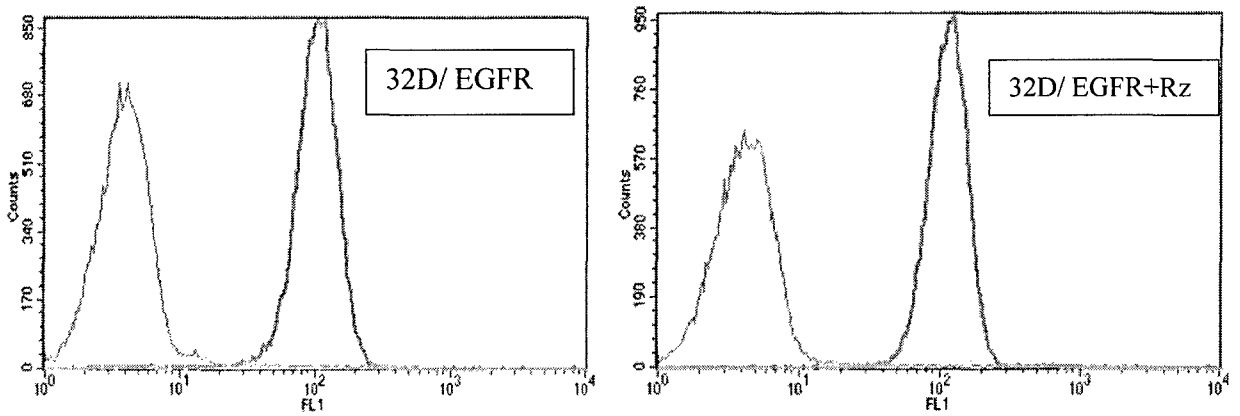


Figure 14B. EGFRvIII ribozyme has no effects on wild-type EGFR protein levels in 32D/EGFR cells. 1x 10^6 cells were used for FACS analysis. The red (most left) curve represents nonspecific staining (primary antibody omitted). The green curves represent the expression levels of EGFR in 32D/EGFR and ribozyme expressing 32D/EGFR (32D/ EGFR+Rz) cells.

Characterization of ribozyme transfected 32D/EGFRvIII cells in vitro: To evaluate whether down-regulation of EGFRvIII will reduce proliferation and reverses the IL-3-independent phenotype. Growth assays were conducted to assess and correlate the EGFRvIII expression levels. Three independent experiments were performed, each in triplicate. Cell number was determined every 2 days, after testing for cell viability by trypan blue exclusion. Values were

reported as the mean of triplicate determinations ± SD. We observed that ribozyme transfected 32D/EGFRvIII cells exhibited IL-3-dependent phenotype as shown in Figure 15. These results suggest that we have generated a biological functional ribozyme, which is capable of down-regulating the EGFRvIII protein levels intracellular and restoring the IL-3-dependent phenotype.

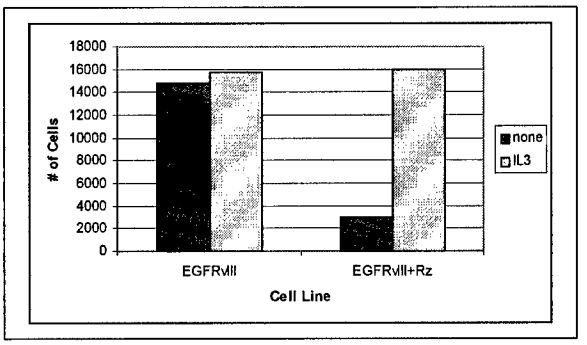


Figure 15. EGFRvIII ribozyme reversed IL-3-dependent phenotype in ribozyme transfected 32D/EGFRvIII cells. 32D/EGFRvIII and ribozyme transfected 32DEGFRvIII cells (EGFRvIII+Rz) were plated at a density of 5 x10^4 cells/ml in IL-3 free medium (none, solid bar), medium supplemented with IL-3 (strike bar). Viable cells were counted on day 3 after seeding. All samples were prepared in triplicate. This assay was repeated more than three times.

Conclusion: We have generated a tumor-specific ribozyme targeting the fusion junction of EGFRvIIImRNA. We demonstrate that this ribozyme has biological functional. This ribozyme efficiently reduced the level of EGFRvIII in the cell. In contrast, the EGFRvIII-ribozyme does not have any effects on wild-type EGFR mRNA and protein levels. Down-regulation of EGFRvIII by ribozyme reverses the IL-3-independent phenotype.

Methods:

1) Ribozyme Construction: Two synthetic single-stranded ribozyme oligonucleotides were subcloned into the mammalian vector pCR3. The sequence and orientation of the inserts were confirmed by dideoxynucleotide sequencing of the construct using the Sequenase kit, version 2.0 (U.S. Biochemical Corp., Cleveland, OH). The EGFRvIII ribozyme sequence is: 5'acauaaucugaugaguccgugaggacgaaacuuucuu 3'. This ribozyme was then subcloned into pCDNA3.1/zeo vector and the sequence and orientation of the inserts were confirmed.

2) Ribozyme-mediated mRNA Cleavage *in Vitro:* The substrate EGFRvIII cDNA fragment was derived by PCR with EGFRvIII full cDNA, which was generously provided by Albert Wong. The PCR primers for subcloning of this EGFRvIII fragment are: 5' primer sequence CCTCCGTCTGAATTTTGCTTT and 3' primer sequences GCCGCGTAGATTTCTAGGTT

We then performed *in vitro* run-off transcripts from an EGFRvIII cDNA construct to generate the EGFRvIII ribozyme substrate. Likewise, EGFRvIII-ribozyme was chemically synthesized as DNA oligonucleotide and subsequently synthesized *in vitro* by using the T7 RNA

polymerase. Cleavage reactions were performed in 50 mM Tris-HCL (pH 8.0) and 20 mM $MgCl_2$, Substrate and ribozyme transcripts were then mixed and incubated at 50°C for 30 min. Reaction products were analyzed on 6% urea polyacrylamide gel and products were detected by autoradiography.

3) Transfection: Cells (1 x 10^6) and 10-15 μg of plasmid DNA were used for each transfection. Transfection was performed using the Calcium Phosphate Transfection System. The cells were then selected in a growth medium containing appropriate amounts of Geneticin (G418-sulfate; Life Technologies).

4) Quantitation of EGFRvIII expression in ribozyme transfected 32D/EGFRvIII cells by FACS analysis: Cells (1 x 10^6) were harvested and then stained for 1 hr with anti-EGFRvIII monoclonal antibody (4-5H) at 4 °C. Stained cells were then washed with cold PBS. A secondary FITC-anti-mouse antibody (CALTAG, CA, USA) was used, and the expression levels of EGFR and EGFRvIII were quantitatively measured by flow cytometry.

5) Evaluation of ribozyme down-regulation of EGFRvIII by Western blotting: Cells were lysed in HEPES lysis buffer (50 nM HEPES, 150mM NaCl, 10% glycerol, 1% Triton X 100, 1.5 mM $MgCl_2$, and 1mM EGTA), and the cell debris was pelleted by centrifugation (7). The lysates were then subjected for immunoprecipitation with anti-EGFRvIII (4-5H), in combination with protein A Sepharose CL-4B (Amersham Pharmacia, Sweden) overnight at 4^0C with gentle agitation. Immunoprecipitates were then separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody (Upstate, Lake Placid, NY), followed by blots with 0.5 ug/ml of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence's reagent (ECL; Amersham Corp.).

6) Growth assays: 50,000 cells/well in IL-3 free medium were plated in 24-well plates (Costar), medium supplemented with IL-3, or medium lacking IL-3 but supplemented with 100ng/ml of EGF were used for this assay. All samples were prepared in triplicate. Three independent assays were performed. Cells were counted in a Coulter Counter (Coulter Electronics LTD, Hialeah, FL) on day 2. Cell counts were reported as the means of triplicate determinations ± SD.

KEY RESEARCH ACCOMPLISHMENTS

- We demonstrate that about 35% (74/208) of prostate cancer specimens express both EGFRvIII and ErbB-2. About 28% (20/74) of these patients detected high levels of expression on both receptors. 32.6% (67/208) of the prostate tumor specimens express either EGFRvIII or ErbB-2 alone and 31% (65/208) of this set of prostate cancer samples was negative on both receptors.
- EGFRvIII is constitutively activated in human prostate cancer patient specimens.
- Expression of EGFRvIII enhances proliferation in both Tsu and DU145 EGFRvIII transfectants.
- Constitutively activated EGFRvIII was detected in prostate cancer transfectants and expression of EGFRvIII in prostate cancer cells induces wild-type EGFR autophosphorylation.
- We also demonstrate that EGFRvIII transfected prostate cancer cells enhance tumorigenicity *in vivo*.
- We have generated a tumor-specific ribozyme targeting the fusion junction of EGFRvIIImRNA.
- We demonstrate that this ribozyme is capable of cleave the EGFRvIII mRNA precisely and efficiently in a cell free system under the physiological condition.
- We also demonstrate that this ribozyme is biological functional. EGFRvIII-ribozyme can efficiently reduce the EGFRvIII expression in an intracellular model system. In contrast, this ribozyme does not have any effects on wild-type EGFR mRNA and protein levels.
- Down-regulation of EGFRvIII by ribozyme reverses the IL-3-independent phenotype.

REPORTABLE OUTCOMES

We have presented our work in the American Association for Cancer Research's 94th Annual Meeting. We also presented this work in CaPCure Prostate Cancer Retreat in August of 2003 at Chicago. I have acknowledged U.S. Army Medical Research and Materiel Command, Prostate Cancer Research Program for the support of my research. Currently, we are preparing a manuscript for publication. We will acknowledge U.S. Army Medical Research and Materiel Command, Prostate Cancer Research Program for the support of my research.

CONCLUSION

We have successfully completed the proposed study and achieved our goal by provide these solid evidences to prove our hypothesis.

Previously, we have detected 44% of prostate tumor specimens express EGFRvIII. In this report, we demonstrate that EGFRvIII is constitutively activated in prostate cancer patient samples. We examined the co-expression of EGFRvIII with ErbB-2 in prostate tumor specimens. Co-expression of EGFRvIII with ErbB-2 was detected in 35% (74/208) of primary prostate cancers. To gain further insight into the role of EGFRvIII in prostate cancer, we expressed EGFRvIII in prostate cancer cells. The expression of EGFRvIII in Tsu and DU145 prostate cancer cell line revealed constitutively activated receptor and induction of wild-type EGFR autophosphorylation. These observations indicate that EGFRvIII could activate wild-type EGFR signaling pathways. Furthermore, expression of EGFRvIII enhances prostate cancer cell proliferation *in vitro* and increases tumorigenicity *in vivo*. We also generated a tumor-specific biological functional ribozyme targeting the fusion junction of EGFRvIII. This EGFRvIII ribozyme appears to be able to down-regulate EGFRvIII expression efficiently, but it does not have any effects on wild-type EGFR expression. Down-regulation of EGFRvIII expression reversed the phenotype in our model system. These results provide the first evidence that EGFRvIII plays a role in human prostate cancer tumorigenesis.

The outcome of this project has allowed us to attain a better understanding of the role of EGFRvIII in prostate cancer tumorigenesis and provided new information about the mutation of EGFR in prostate cancer tumorigenesis. These results may eventually lead to intervention in diagnosis, prognosis and treatment of prostate cancer.

We also would like to take this opportunity to appreciate the support from U.S. Army Medical Research and Materiel Command, Prostate Cancer Research Program, which facilitates this novel research.

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Abstract for AACR 94th Annual Meeting

Biological Role of EGFRvIII in Human Prostate Cancer

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EGFRvIII is a tumor specific, ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has been detected in gliomas and various other human malignancies, including prostate cancer. In order to evaluate the importance of EGFRvIII expression in human prostate cancer, we examined the frequency of EGFRvIII protein expression immunohistochemically in a prostate tissue microarray, which consists 206 patient's tissues with prostate cancer. With a specific anti-EGFRvIII antibody, we detected 40% of primary prostate carcinomas expressed EGFRvIII. However, no EGFRvIII expression was detected in normal prostate tissues. With this specific anti-EGFRvIII antibody, we also detected that EGFRvIII was constitutively activated in prostate cancer tissues. These results suggest that EGFRvIII may play a crucial role in prostate cancer progression.

To delineate the biological significance of EGFRvIII in human prostate cancer, we expressed EGFRvIII in Tsu human prostate cancer cells. Expression of EGFRvIII in Tsu cells produced a constitutively activated EGFRvIII receptor. Interestingly, the expression was detected in the cytoplasmic, but not on the cell membrane. These Tsu/EGFRvIII transfectants exhibited increasing proliferation and induced colony formation in anchorage-dependent and anchorage-independent assays. Collectively, these results provide the evidence that EGFRvIII could play a role in human prostate cancer progression.

Abstract for CaPCure Tenth Annual Scientific Retreat Nov. 8 – Nov. 10, 2003 in New York, NY.

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To delineate the biological significance of EGFRvIII in human prostate cancer, we expressed EGFRvIII in DU145 human prostate cancer cells. Expression of EGFRvIII in DU145 cells produced a constitutively activated EGFRvIII receptor. These DU145/EGFRvIII transfectants exhibited increasing proliferation in an anchorage-dependent assay. Collectively, these results provide the evidence that EGFRvIII could play a role in human prostate cancer progression.